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Detection and identification of adherence genes of intestinal-origin *Lactobacillus* and *Pediococcus* strains grown on gastric mucin *in vitro*

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Abstract

One of the primary selection criteria for potential probiotics is the ability to adhere to the host gastrointestinal tract. This study evaluated the *in vitro* adhesion ability on gastric mucin of two *Lactobacillus casei* strains (AP and AG) and two *Pediococcus acidilactici* strains (BE and BK), and identified the corresponding genes responsible for adherence. Adhesion assays were performed in 96-well polystyrene microtiter plates using gastric mucin from porcine stomach as the matrix. An *in vitro* study on gastric mucin revealed that lactobacilli had a greater adherence ability compared with pediococci strains. The potential adherence genes were investigated using polymerase chain reaction (PCR) technology. Using specific primers, PCR studies amplified 150 base pairs of a potential *mub* gene and 161 base pairs of a potential *ef-Tu* gene, but no amplified bands for potential *map* and *bac* genes were obtained. Sequence comparisons showed that the 150 and 161 amplified base pairs were respectively homologous to the *mub* of *Pediococcus acidilactici* and *ef-Tu* genes of *Lactobacillus paracasei*. We concluded that the adherence ability of two strains of *Lactobacillus casei* (AP and AG) and two strains of *Pediococcus acidilactici* (BE and BK) on gastric mucin is in accordance with the presence of *ef-Tu* and *mub* genes. High level attachment in lactobacilli is likely to correlate with the *ef-Tu* gene, which is a lactobacilli-specific adhesive gene.

Keywords: Adherence genes, intestinal origin, Lactic Acid Bacteria

Introduction

Bacterial strains for probiotics have been isolated from different sources; and one of the best sources is the gastrointestinal tract (GIT) of humans (Margolles *et al*., 2009). It has previously been reported that the rapid development of probiotics inside the human GIT is due to the consumption of human milk oligosaccharides (HMOs) (Favier *et al*., 2003). In order to function as probiotics, bacterial strains must have specific criteria. One of the important criteria is the ability of strains to attach to gut epithelial tissues and colonize the GIT (Collins *et al*, 1998; Dunne *et al*., 1999). Adherence to the mucosa of the digestive tract allows probiotic colonization, immune stimulation and competition with pathogens by means of competition for receptor sites at the surface of the host intestine (Lahtinen and Ouwehand, 2009). Probiotic colonization of intestinal surfaces prevents the presence of pathogens, and consequently protects the host from bacterial infection (Brassart and Schiffrin, 2007).

The ability to bind to the GIT, along with survival in the intestine, is generally considered to be the main characteristics observed in probiotics. Previous studies on the molecular basis of adhesion have been conducted mainly based on the *in vitro* adhesion of probiotics cells to adhesion models such as cell lines (Caco-2 and HT-29) or mucus preparations (Ljungh and Wadstrom, 2009). A number of adhesion

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proteins have been studied and characterized, including mucus-binding proteins (Mub) (Roos and Jonsson, 2002), mucus adhesion promoting protein (MapA) (Ramiah *et al*., 2007), anchorless multifunction proteins elongation factor Tu (Ef-Tu) (Greene and Klaenhammer, 1994; Ramiah *et al*., 2007) and surface layer proteins (Boot *et al*., 1993; Vidgren *et al*., 1992). According to Ljungh and Wadstrom (2009), proteins involved in the adhesive mechanism can be separated into five classes: anchorless housekeeping proteins, surface layer proteins, LPXTG-motif proteins, transporter proteins, and other proteins. The corresponding genes for these proteins has also been investigated, including *ef-Tu*, *bac*, *map*A, *mub*, *msa*, and *slp*A (Turpin *et al*., 2012). The binding capacity of probiotics varies between strains, and could be due to differential expression of the corresponding genes. At least 20 genes were reported to be functionally important in the binding of *Lactobacillaceae* to the digestive tract (Turpin *et al*., 2012).

Widodo *et al.* (2012a; 2012b) successfully identified *Lactobacillus casei* strains AF, AP, and AG and *Pediococcus acidilactici* strains AA, BE, and BK in the feces of Indonesian infants consuming breast milk. Of these strains, *Lactobacillus casei* strains AP and AG, and *Pediococcus acidilactici* strain BE were able to degrade prebiotic inulin (Widodo *et al*., 2012b; 2014) and showed potential as probiotics. However, the ability of these strains to attach to the GIT has never been investigated. This study was carried out to investigate the adherence ability of selected strains *in vitro*, and to identify adherence genes that might correlate with the ability to attach to intestinal surfaces.

Materials and Methods

Bacterial strains and adherence assays in vitro

Lactobacillus casei strains AP and AG and *Pediococcus acidilactici* strains BE and BK were obtained from previous experiments (Widodo *et al*., 2012a; 2012b). Bacterial strains were routinely cultured at 37 °C in de Man Rogosa Sharpe (MRS, Merck) media. *In vitro* adherence ability assay was carried out according to Sanchez *et al*. (2010) and Roos and Jonsson (2002) with modification. Adhesion assays were performed in 96-well polystyrene microtiter plates (Corning) using gastric mucin from porcine stomach (Sigma) as the matrix.

Genomic DNA extraction

Genomic DNA was extracted from 1.5 mL of overnight cell culture, suspended in 400 µL SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5) containing lysozyme (30 mg/mL). The solution was mixed by inverting the tube, and incubated at 37 °C for 1 h. Fifty (50) µL of SDS solution (10%) was added and followed by incubation at 65 °C for 1 h. After 1 h of incubation, 167 µL of NaCl solution (5M) was added and incubation was prolonged for another 1 h. DNA was separated from cellular debris with 400 µL chloroform, incubated at room temperature for 10 min, and then centrifuged at 13000 rpm for 10 min. The top aqueous phase was transferred to a new Eppendorf tube and the DNA was precipitated overnight with isopropanol at −20 °C. Precipitated DNA was harvested by centrifugation at 13000 rpm for 10 min, washed with 500 µL ethanol (70%), and resuspended in Tris-EDTA (TE) buffer. Agarose gel electrophoresis was carried out on 0.8% agarose gel in TAE buffer at 80 V for 1 h. Size was calculated using a 1 kb DNA Ladder (Promega, USA).

DNA amplification

Amplification of the gene of interest was carried out using PCR with primers designed according to Ramiah *et al.* (2007), as presented in Table 1. The glyceraldehyde 3-phosphate dehydrogenase (GDPH) gene was used as the standard reference gene. Gene amplification was performed using a PCR thermal cycler, with the protocol set as follows: denaturation at 95 °C for 30s, annealing at 56 °C for 30s, elongation at 72 °C for 45s (30 cycles), and a final extension

Table 1. Primers for PCR amplification.

Primers	Sequence 5′ to 3′	Amplicon (base pairs)
EFTu	F (TCGATGCTGCTCCAGAAGAAA) R (TGGCATAGGACCATCAGTTGC)	161
Mub	F (GTAGTTACTCAGTGACGATCAATG) R (TAATTGTAAAGGTATAATCGGAGG)	150
Bac	F (GACATTTACATTGAGTAGGAACTAG) R (GTAACCCCATTACCATAGTATTTAC)	147
Map	F (TAATTGTAAAGGTATAATCGGAGG) R (TGGATTCTGCTTGAGGTAAG)	156
GDPH	F (ACTGAATTAGTTGCTATCTTAGAC) R (GAAAGTAGTACCGATAACATCAGA)	140

at 72 °C for 10 min. Amplified bands were resolved by electrophoresis in 1.6% (w/v) agarose gels and visualized using ethidium bromide staining.

DNA sequencing and phylogenetic analysis

The amplified DNA was sequenced using an Applied Biosystems 3730-XL Analyzer at 1st Base Sequencing, Kuala Lumpur, Malaysia. The resulting sequences were used to search the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) algorithm. The isolates were identified on the basis of the sequences that produced the best match (>97%). Molecular Evolutionary Genetics Analysis (MEGA) 6.0 was used to construct a phylogenetic tree of the sequences based on the neighbor-joining algorithm (Saitou and Nei, 1987).

Results and Discussion

Some of the beneficial effects exerted by probiotics are triggered by the interaction with the gastrointestinal (GIT) mucosa. As such, adhesion to the GIT mucosa is the most crucial factor contributing to successful probiotic colonization in upper intestinal regions (Tannock, 1992). Mucosal colonization by probiotics can be found both in the mucus layer and epithelial cells (Brassart and Schiffrin, 2007). The epithelial cells of the intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids, with large glycoprotein mucin being the main component. Complexes of glycoproteins in the mucus layer prevent the host from harmful antigens and pathogens, and promote luminal motility (McGroarty, 1993; Van Tassell and Miller, 2011). Therefore, adhesion to this mucus is required for probiotic interaction to occur with the host and to elicit a response (Van Tassell and Miller, 2011). Given that mucin is the main component of the mucus layer, adhesion assays were performed *in vitro* using gastric mucin from porcine stomach (Sigma) as the matrix. The use of *in vitro* models, as opposed to *in vivo* ones, was due to the complexity of *in vivo* studies.

Table 2 shows that every strain had a different ability to adhere to gastric mucin *in vitro*. Lactobacilli strains (AP and AG) had a greater adherence ability compared with pediococci strains (BE and BK). The percentage of adhering cells of strains AP, AG, BE, and BK on mucus after 60 minutes were 49.25±0.35%, 53.03±0.48%, 36.80±0.46%, and 23.96±0.32, respectively.

The high adhesion capability in lactobacilli presented here is in agreement with previous findings. A study by Drury *et al*. (2011) demonstrated the ability of indigenous probiotic *Lactobacillus* strains Lp9 and Lp91 to adhere to Caco-2 and HT-29 colonic adenocarcinoma human intestinal epithelial cell lines *in vitro*. They reported that *Lactobacillus* Lp91 was the most adhesive strain to HT-29 and Caco-2 cell lines with adhesion values of 12.8% and 10.2%, respectively. Another study by

Table 2. *In vitro* adhesion assays of four probiotic strains on mucin.

Strains	Time of incubation (minutes)		Percentage of adhering cells after 60 minutes (%)
	0	60	
AP	199.5 ± 0.58	98.25 ± 0.50	49.25 ± 0.35
AG	177.25 ± 0.50	94.00 ± 0.82	53.03 ± 0.48
BE	146.75 ± 0.50	54.00 ± 0.82	36.80 ± 0.46
BK	144.00 ± 0.82	34.50 ± 0.58	23.96 ± 0.32

AP: *Lactobacillus casei* strain AP; AG: *Lactobacillus casei* strain AG; BE: *Pediococcus acidilactici* strain BE; and BK: *Pediococcus acidilactici* strain BK.

Lewandowska *et al*. (2005) demonstrated an adhesion yield of 33.81% for *Lactobacillus rhamnosus* GG, and 3.89% for *Lactobacillus helveticus*, to Caco-2 cells. Meanwhile, a study by Xu *et al.* (2009) demonstrated a less than 10% adhesion yield of *Pediococcus acidilactici* KACC 12307 to Caco-2 cells.

The high adherence ability of *L. casei* strains AP and AG *in vitro*, as presented in Table 2, is unlikely affected by the presence of the *mub* gene because the identified *mub* gene in this study is highly homologous with the relevant genes from non-*L. casei* species, namely *P. acidilactici* and *L. plantarum*. Mub was the first *Lactobacillus* adhesion protein shown to contain the same domain organization as that of adhesins from other Gram-positive bacteria (Ramiah *et al*., 2007). Mub proteins have a high molecular weight (358 kDa) with approximately 200 amino acid long sequences and regions typical for other cell surface proteins in Gram-positive bacteria, such as an N-terminal secretion signal peptide, a cell wall anchoring motif (LPXTG), a putative membrane-spanning region and a cell-membrane anchor. The *Mub* gene is expressed 80–140-fold more in the presence of mucin, but is suppressed 7–30-fold under normal gut physiological conditions containing bile and pancreatin (Ramiah *et al*., 2009).

The ability of the four strains to adhere on gastric mucin *in vitro* (Table 1) might correlate with the presence of the *ef-Tu* gene (Figure 1). The Ef-Tu protein facilitates the transfer of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis (Gaucher *et al*., 2001). However, the Ef-Tu protein also binds to mucin and mediates bacterial colonization of human intestinal cells and mucus.

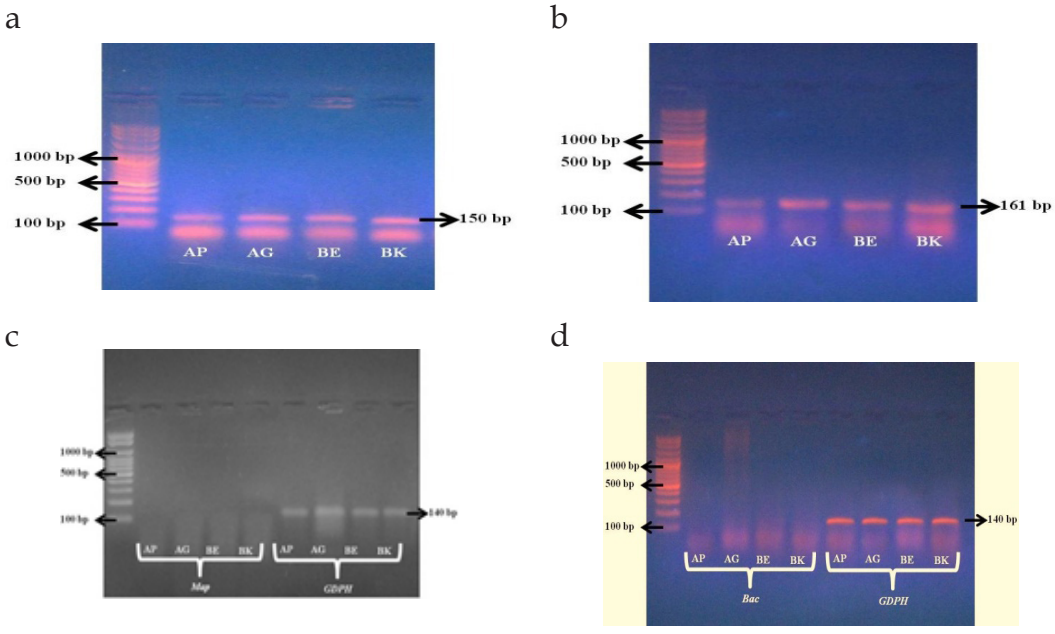

Figure 1. Amplification of (a) *mub*, (b) *ef-Tu*, (c) *map*, and (d) *bac* genes.

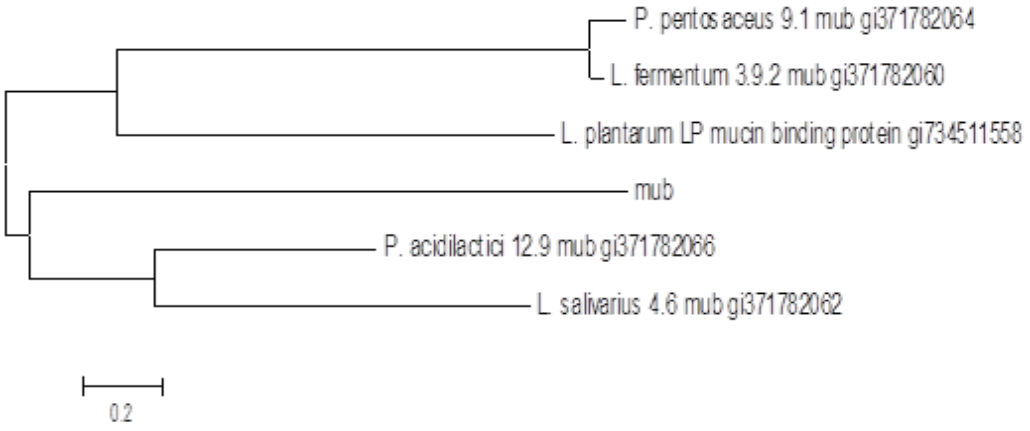

Figure 2. Phylogenetic tree showing the genetic relationship of the *mub* gene of the selected strains with other *mub* genes from genera *Lactobacillus* and *Pediococcus* based on 16S rRNA sequences.

The binding of e*f-Tu* to mucus or cells has been proposed to have a role in gut homeostasis (Granato *et al.*, 2004). The *ef-Tu* gene is expressed 33–100 times higher in media containing mucus, but is not affected by bile or pancreatin concentrations (Ramiah *et al.*, 2009). This may indicate an interplay between different mechanisms regulating adhesin expression to adapt to particular environments.

Figure 1 shows that the *mub* and *ef-Tu* genes were detected in all of the strains (AP, AG, BE, and BK), as seen with the presence of amplified 150 and 161 base pair (bp) sequences, respectively, suggesting that all the strains carry the *ef-Tu* and *mub* genes. In contrast, the other two genes (*map* and *bac*) were not detected in any of the strains, indicating that none of the four strains carry these genes (Figure 1).

Further elaboration of the amplified 150 bp was carried out. The amplified 150 bp was isolated, purified, and sequenced. The sequence comparison of the selected bands with a relevant database showed that the amplified 150 bp was highly homologous with the partial *mub* gene of *Pediococcus acidilactici* 12.9 (Figure 2). Meanwhile, the amplified band of 161 bp of the candidate *ef-Tu* gene was also investigated. The amplified 161 bp bands were sequenced, and the sequence data were compared with a relevant database *in silico*. The sequence comparison showed that the amplified 161 bp band showed high similarity with the sequence of the *ef-Tu* gene from *Lactobacillus paracasei* strain LBS3 (Figure 3). Figure 3 shows that the *ef-Tu* gene was genetically close to other *ef-Tu* genes from lactobacilli, suggesting that the higher level of adherence on lactobacilli than on pediococci is in line with the presence of *ef-Tu* genes.

Based on molecular identification, this study also reports that none of the four strains carry the *map* and *bac* genes (Figure 1). A mucus adhesion promoting protein (map) is a cell surface protein with a molecular mass of 26 kDa and theoretical pI of 9.7 (Ramiah, 2007). The *map*A gene is upregulated 6–8-fold when incubated in the presence of mucin and up to 25-fold when exposed to physiological concentrations of pancreatin and bile compared with MRS grown controls (Ramiah *et al.*, 2009). A previous study also found that the *map*A gene is significantly down-regulated in the presence of cysteine, suggesting that cysteine is an effector molecule that represses transcription of the *map*A gene. Similar to the *map* gene, none of the tested strains carry the *bac* gene. According to Ramiah (2009), the *bac* gene is highly detected in the genome of *Lactobacillus plantarum* 423 in ileum and cecum tissue samples. These data suggest that not all lactic acid bacteria have the *bac* gene, and the presence of this gene is tissue-specific.

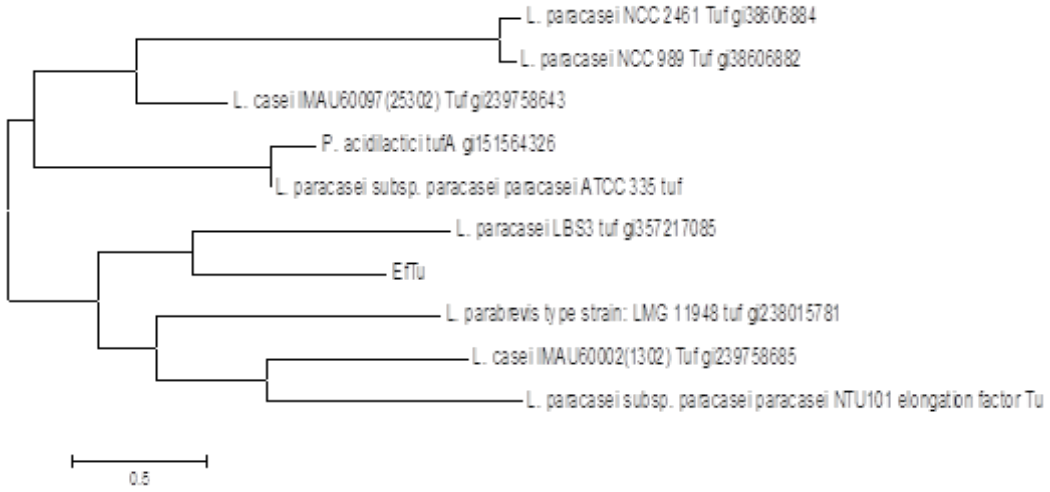

Figure 3. Phylogenetic tree showing genetic relationship of the *ef-Tu* gene of the selected strains with other *ef-Tu* genes from genera *Lactobacillus* and *Pediococcus* based on 16S rRNA sequences.

Conclusion

The *in vitro* adherence ability of two strains of *Lactobacillus casei* (AP and AG) and *Pediococcus acidilactici* (BE and BK) is in accordance with the presence of *ef-Tu* and *mub* genes but not *map* and *bac* genes. High level attachment in lactobacilli is likely to correlate with the *ef-Tu* gene, which is a lactobacilli-specific adhesive gene.

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